Evaluation of Anthelmintic Activity of Herbal Nanoformulations Against Gastrointestinal Nematode, *Heamonchus contortus*

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Haemonchus contortus (Rud., 1803) is the most significant and common parasite in the sheep and goat of the tropical, sub-tropical and temperate countries of the world. It is the highly pathogenic gastrointestinal nematode of the small ruminants. The adult parasite is attached to the gut of host and feeds upon blood in the stomach (abomasum). It causes anaemia, haemorrhagic gastroenteritis, edema (bottle jaw), diarrhea and depression of mineral level, all contributing to weight loss, reduced milk, meat and wool production and frequent death. Haemonchosis is a hyper acute disease, as death of the ruminants occurs within one week without significant signs resulting in great production and the economic losses in the countries where animal husbandry is important occupation.

Conventional anthelmintics and pasture management are widely used strategies to fight against this pathogenic gastrointestinal nematode. There are various drugs which are used against them like Fenbendazole, Mebendazole, Thiabendazole, Levamisole and Ivermectin however, wide use of these anthelmintic drugs led to the development of resistance in *H. contortus* (Singh *et al.,* 2002; Chandra *et al.,* 2014). Parasite strains resistant to one group of drugs may also be resistant to other groups, which suggest the existence of multiple drug resistance to the major anthelmintic drugs. Increase of resistant of parasitic strain causes high cost, reduction of animal production and risk of environmental pollution that has arose interest in medicinal plant and plant product as an alternative source of anthelmintic (Herd, 1996; Waller, 1997; Melo *et al*., 2003). The herbal extracts are biodegradable and non toxic to the environment as well as the livestock hence, are used to control these parasite.

Researchers have explored the use of plants to control barber pole worm. Feeding of *Sericcea lespedeza* hay to goats can reduce fecal eggs counts by 80 percent and create a higher packed cell volume. *Sericcea lespedeza* a perennial legume native of Eastern Asia and contain condensed tannins. The number of stomach worms and egg production reduced by condensed tannins (Shaik *et al*., 2004; Lange *et al.*, 2006). Besides using the whole plant, their extracts also act as potent anthelmintic. There are various medicinal plants screened by several researchers and assessed for their anhelmintic properties (Jabbar *et al.,* 2006).

In the recent years, nanotechnology has attracted considerable interest of researchers all over the world because of the peculiar physical, chemical and biological properties of nanopeprations. Among them silver nanoparticles and nanoemulsions have been evaluated for their biological properties like antifungal, biomedical, insecticidal, antiviral, antimicrobial activity etc. A number of methods have been reported for the synthesis of silver nanoparticles including chemical, physical and biological procedures. However, the chemical and physical methods require the use of toxic chemicals, high pressure, temperature and energy. Therefore, biologically synthesized nanoparticles are more promising over the chemical and physical method since it is eco friendly and economic method. Various plants were screened for green synthesis of silver nanoparticles like *Azadirachta indica* (Shankar *et al.,* 2004; Tomar and Preet, 2016), *Aloe vera* (Chandran *et al.,* 2006).

Nanoemulsions are the thermodynamically stable, optically clear, translucent or transparent heterogeneous system constituted by two immiscible liquids where one phase is oil phase while other is aqueous phase and one or more surfactants, with small droplet size (20-200 nm). Surfactants such as tween 80, Capryol 90, Poloxamer 124 and 188, Softigen 701, 767, Lauroglycol 90 and Cremophor RH 40. The stable nanoemulsions can be produced by both high-energy and low-energy methods. High energy methods involve high pressure homogenization (HPH) and ultrasonications whereas, low-energy emulsification methods depend on the properties of the constituents and phase behaviour, and they utilize the stored energy of the system to form nano droplets. There are only a few reports where nanoemulsions have been used as anthelmintic (Grando *et al.,* 2015; Ribeiro *et al.,* 2015).

Nanoencapsulation is a technique through which small amount of particle of required materials are packed within a unique polymer membrane to form capsules of nanoscale range (Lopez *et al.,* 2006). Nanoncapsulation technology was employed to safe guard of bioactive compounds (micronutrients, polyphenols, enzyme, herbal products, and antioxidants) and to protect them from adverse environmental conditions and also for the controlled release at targeted sites (Gouin, 2004). Most recently, nanoencpsulated oil have been fabricated and evaluated for their anthelmintic potential (Ribeiro *et al.,* 2014).

Literature survey clearly indicates trace studies, where nano based formulations have been tested for their anthelmintic potential. Moreover, there is a growing concern regarding the

toxicity issues with the extreme use of nanometerials in diverse fields. Therefore, toxicity assessment would be a prerequisite for the development of effective anthelmintic through the use of nanotechnology. Hence, the present study would focus on the synthesis and characterization of herbal nanoformulations and their comparative anthelmintic effectiveness against *H. contortus.* Further, the work would be extended to evaluate cytotoxicity of these formulations on selected mammalian cell lines to develop a safe herbal nano based anthelmintic for safe guarding the livestock.

LITERATURE REVIEW

Gastrointestinal parasites severely affect the livestock production in tropical, sub-tropical and developing countries of the world, (Hounzangbe-Adote *et al*., 2005) particularly in the regions where extensive grazing is practiced (Waller 1997). It causes large economic losses to livestock breeders by causing damages in gastric function i.e. gastritis, anaemia and associated complications, appetite depression, leading to production losses and death in severely affected animals.

In order to overcome this problem several methods have been used. The most common conventional method to control gastrointestinal nematodes is the use of chemical anthelmintics. The widespread and excessive use of chemical anthelmintics has resulted in drug resistance. Drudge *et al.* (1964) accurately described the first case of resistance to anthelmintics. After that several studies reported the anthelmintic resistance to all three broad spectrum families of anthelmintics *viz.* Benzimidazole, lmidazothiazole and Ivermectin against gastrointestinal parasite (Rolfe *et al*., 1990; Swarnkar *et al*., 1999; Chandra *et al.*, 2015). Therefore, anthelmintics derived from plants can be an environmentally safer alternative measures for the treatment of gastrointestinal infections (Akhtar *et al.*, 2000; Kamaraj *et al.*, 2011).

Chandrawathani *et al.* (2000) reported feeding of fresh *Azadirachta indica* leaves produced 82% fecal eggs reduction count. The 62% reduction of the abomasal nematode *H. contortus* egg counts resulted with the consumption of the whole plant extract of *Artemisia brevifolia* (Iqbal *et al*., 2004). Hounzangbe-Adote *et al*. (2005) studied the consumption of fagara leaves (*Zanthoxylum zanthoxyloides*), a native tree from Africa, believed to have antiparasitic activity, when consumed regularly in small amounts, resulted in reduced egg count of *H. contortus* in sheep.

Methanolic extracts of *Allium sativum, Zingiber officinale, Curcurbita mexicana* and *Ficus religiosa* having *in vitro* larvicidal potential against nematode parasite was reported by Iqbal *et al.* (2001**)**. Chandrawathani *et al.* (2006) assessed the anthelmintic effect of *Azadirachta indica* on *H. contortus* parasites of sheep. *In vitro* and *In vivo* anthelmintics activity of crude extracts of *Coriandrum sativum* against gastrointestinal nematode was reported by Eguale *et al.* (2007). *Costa et al. (2008)* tested *In vitro* ovicidal and larvicidal activity of *Azadirachta indica* ethyl and ethanol extracts on *H. contortus*. Embryonicidal, ovicidal and larvicidal potential activity of extracts of *Butea*

frondosa against exogenous stages of *H. contortus* was reported by Swarnkar *et al.* (2008). The crude extracts of *Maesa lanceolata* and *Plectranthus punctatus* exhibited ovicidal and larvicidal activity against *H. contortus* as reported by Tadesse *et al. (2009). In vitro* anthelmintic activity of aqueous extracts of *Leonotis leonurus, Aloe ferox* and *Elephantorrhiza elephantina* against *H. contortus* was reported by *Maphosa et al. (2010).* During the same year Kamaraj *et al.* (2010) reported ovicidal and larvicidal activity of crude extracts of *Melia azedarach* against *H. contortus* (Strongylida). They also tested (Kamaraj *et al.*, 2011) bioefficacy of *Annona squamosa, Eclipta prostrate, Terminalia chebula, Catharanthus roseus and, Solanum torvum* extracts against *H. contortus.* Carvalho *et al.* (2012) reported anthelmintic potential of plant extracts on *H. contortus* and *Strongyloides venezuelensis. In vitro* anthelmintic activity of crude extracts of some medicinal plants against *Haemonchus contortus* from sheep was demonstrated by Ahmed *et al.* (2013). The toxicity and efficacy study of thirteen plant leaf acetone extracts on egg hatching and larval development of *H. contortus* was reported by Adamu *et al.* (2013).

Recently, Shahjahan *et al.* (2015) studied *In-vitro* anthelmintic efficacy of some Kashmiri medicinal plants against *H. contortus*. *In-vitro* larvicidal potential of *Vernonia amygdalina* roots using adult *H. contortus* worms was demonstrated by Sirama *et al.* (2015). *In vitro* Evaluation of few indigenously known herbal plants for their anthelmintic property was studied by Chander *et al.* (2016). Calvin *et al.* (2016) reported *In vitro* anthelmintic activity of aqueous extract of *Crassocephalum crepidioides* (Benth.) on *H. contortus.* Anthelmintic activity of selected ethno-medicinal plant extracts on parasitic stages of *H. contortus* was assessed by Kumarasingha *et al.* (2016). Fouche *et al*. (2016) studied anthelmintic activity of acetone extracts from South African plants for evaluated egg hatching of *H. contortus.*

Besides using the herbal extract, many researchers have reported the anthelmintic potential of plant essential oils on *H. contortus*. Modulating ruminal fermentation to improve nutrient utilization due to this essential oil has been used in nutrition and also as phytotherapy in ruminants (Hristov *et al.*, 1999). Macedo *et al.* (2010) studied Anthelmintic effect of *Eucalyptus staigeriana* essential oil against *H.contortus. In vitro* Anthelmintic activity of *Cymbopogon martinii*, *Cymbopogon schoenanthus* and *Mentha piperita* essential oils against gastrointestinal nematode was demonstrated by Katiki *et al.* (2011). *In vitro* ovicidal and larvicidal activity of the essential oil of *Artemisia lancea* against *H. contortus* was demonstrated by Zhu *et al.* (2013).

Nanoemulsions are considered suitable for efficient delivery of active ingredients through the biological membrane. The large surface area of the nanoemulsion permits rapid penetration of bioactive component. Due to this reason nanoemulsions are used in medical science. There are the various essential oils are used to make nanoemulsions and few are tested against *H.contortus.* Anjali *et al.* (2011) prepared stable neem oil nanoemulsion with droplet size of 31.03 nm at 1:3 ratio of oil and surfactant (Tween 80). They also tested their larvicidal potential on *Culex qninquefasciatus*. Grando *et al.* (2015) formulated the leaves of the native Australian plant *Melaleuca alternifolia* popularly known as 'tea tree oil' nanoemulsion and tested their *In-vitro* anthelmintic activity against *H. contortus.* Jayakumar *et al.* (2015) formulated the neem oil nanoemulsions (1:3 ratio) with droplet size in the range of 30–70 nm. Tween 20 was used as the surfactant for the formulation. They tested their antibacterial properties against bacterial pathogen *Vibrio vulnificus* and also studied cellular toxicity of formulated nanoemulsion on human lymphocytes. Ribeiro *et al.* (2015) formulated nanoemulsion from *Eucalyptus staigeriana* essential oil of mean particle size was 274.3 nm. They tested their anthelmintic activity and also tested their toxicity on mice.

Nanoencapsulations are also being fabricated due to their effective drug delivery to the target cell. Ribeiro *et al.* (2013) formulated the chitosan-encapsulated *Eucalyptus staigeriana* essential oil and tested their anthelmintic potential on gastrointestinal nematode. Efficacy of free and nanoencapsulated *Eucalyptus citriodora* essential oils on sheep gastrointestinal nematodes was studied by Ribeiro *et al.* (2014). Besides nanoemulsions and nanoencapsulations biological synthesis of nanoparticles has received increased attention due to a growing need to develop environmentally benign technologies in material synthesis. Several plant species have been screened in this regard and only a few have been reported for their antiparasitic potential (Marimathu *et al*. 2011; Said *et al.* 2012). However, only one report exists till date on their anthelmintic properties of against *H. contortus* (Tomar and Preet 2016). Silver nanoparticles contain free ions which possess some toxic effect on the physiology of the organisms. There are only few studies in which the toxic effects of the nanoformulation have been studied. Ribeiro *et al.* (2014) studied toxic effect of nanoencapsulated *Eucalyptus citriodora* essential oils on mice. Milic *et al.* (2014) synthesized silver nanoparticles through chemical method and tested their toxicity on mammalian kidney cells.

Literature survey clearly indicates only recent trace studies with nanopreparations for controlling gastrointestinal nematode. Hence, the present study would involve a comparative study for evaluating the effectiveness of diverse nanoformulations and the work would be extended to assess their cytotoxic effect on selected mammalian cell lines for developing a safe herbal anthelmintic.

OBJECTIVES

- Synthesis and characterization of herbal nanoformulations.
- Assessment of *In-vitro* adulticidal potential of nanoformulations against *Haemonchus contortus*.
- Evaluation of ovicidal potential of nanoformulations on *Haemonchus contortus* through egg hatch essay.
- Assessment of cytotoxicity of nanoformulations in selected mammalian cell lines through MTT assays.

MATERIALS AND METHODS

1. MATERIALS:

Experimental Animal: *Haemonchus contortus*

Taxonomic Position

Kingdom - Animalia
Phylum - Nematoda
Class - Secernentea
Sub-class - Rhabditia
Order - Strongylida
Family - Trichostrongylidae
Genus - *Haemonchus*
Species - *contortus*

Haemonchus contortus, (Barber's pole worm) is the most abundant and pathogenic gastrointestinal nematode of ruminants. Adult worms feed on the blood and attached to abomasal mucosa (stomach). This parasite mainly infects the ruminants during summer months in warm, humid climates and it causes anaemia, diarrhea, bottle jaw and death of infected ruminants.

Morphology:

The adult *H. contortus* female is red and white in appearance because the white ovaries that coil around intestine which is filled with blood. The adult female is 18–30 mm long and the male adult worm is much smaller (10–20 mm) than the female and has well-developed copulatory bursa. The eggs are yellowish measuring about 70–85 μm X 44 μm.

Life cycle

The life cycle is simple small ruminant become infected when they eat grasses containing the L3 larvae (infective stage) of *H. contortus*. The infecting larvae reach the abomasums within 48 hours. L-3 larvae shed their cuticle and burrow into the abomasal mucosa where they develop into L-4 or pre-adult larvae. L-4 larva then molts and develops into adult form. The male and female adults feed upon the blood where they mate and live in the abomasum. The adult female worm can release approximately 5,000 – 10,000 eggs in the feces. Eggs then develop into the L1 (rhabditiform), and L-2 juvenile stages in moist conditions (temp. 24-25°C) in the faeces and feed on bacteria in the dung.

The L-3 filiariform infective larvae are developed when L-2 rhabditform larvae sheds their cuticle. The L-3 infecting stage then crawl up the blades of wet grass and wait to be ingested by a grazing animal.

METHODOLOGY:

2.1 Collection of Sample:

The worms of *H. contortus* will be collected from the abomasum of freshly slaughtered goats. The abomasum will be collected from slaughter house of Agra. Immediately after slaughter, the abomasum will be collected and transported to the laboratory in Phosphate buffer saline (PBS). The parasites will be obtained from abomasum, washed and kept in PBS.

2.2 Synthesis of nanoformulations:

Various nanoformulations will be prepared and then anthelmintic potential would be evaluated against *H. contortus.* Firstly, biological synthesis of silver nanoparticles (AgNPs) would be accomplished and for this various plants will be screened individually and in combinations. The Fresh leaves of selected plant will be washed with purified distilled water and cut into small pieces. A known amount of leaves (to obtain 5-25% aqueous herbal extract) will be added to 100ml of deionized water and heated. The extract would be filtered through Whatman no. 1 filter paper. The resultant aqueous extract will be interacted with freshly prepared silver nitrate (1mM) solution. The periodic sampling of aliquots through ultraviolet–visible (UV-Vis) spectrophotometry in 300-700 nm range would confirm the reduction and synthesis of AgNPs. The broth containing AgNPs will be centrifuged at 10,000rpm for 15 minutes and redispersed in distilled water to get rid of impurities and purified pellets would be dried.

Secondly, nanoemulsions would be synthesized by using high energy method i.e. ultrasonication. Some Essential oil (with known active component) of medicinally important plant like Ginger oil, lemon oil etc, polyoxyethylene sorbitan monooleate (Tween 80) and Milli Q water will be used for the preparation of nanoemulsions. Tween 80 used as the surfactant or emulsifier as it is the non toxic, hydrophilic, stable against pH variation and ionic strength and compatibility with other ingredients. Oil and surfactant will be mixed in different ratio to optimize the protocol for nanodroplet size. The resultant emulsion will be sonicated for 1h with the help of ultrasonicator.

Thirdly, nanoencapsulation will be prepared using essential oils. Essential oils of Lemon (*Citrus limonum*, Family- Rutaceae), Ginger (*Zingiber officinale*, Family -Zingiberaceae) and Garlic oil (*Allium sativum*, Family - Amaryllidaceae) will be used for nanoencapsulations preparation and subsequent evaluation of their anthelmintic properties. Essential oils are concentrated hydrophobic liquids containing highly concentrated natural volatile aroma constituents from plants. These oils will be obtained from different parts of selected plants such as lemon rind, ginger rhizome and garlic bulb. Air-dried plant materials of different selected plants will be used and subjected to hydro distillation for 4h using a Clevenger-type apparatus. The obtained oils will be dried over hydrous sodium sulfate for 24h, filtered and will be stored at 4 ºC in brown sealed glass vials until used (Zhu *et al.,* 2013). Nanoencapsulation will be prepared with essential oils by mixing them with Tween 80 at various ratios to optimizing the protocol and sonicated by using ultrasonication for 1hours. This mixture will be added with 1% chitosan solution under ultrasonication for 10 min. Chitosan will be used as the encapsulating matrix for their biodegradable and non-toxic nature. The resultant mixture would be allowed to stand for 10 min at 27°C temperature. The macroscopic characteristics of essential oils stability will be observed after 72 h to monitor for a possible phase separation (Ribeiro *et al.*, 2014).

2.3 Characterization of nanoformulations:

The characterization of biofabricated nanoformulations will be done with the help of Scanning Electron Microscopy (SEM) for the surface topography, X-ray diffraction (XRD) for the crystalline nature of the nanoparticles, Transmission Electron Microscopy (TEM) for determining the shape and size of the synthesized nanoformulations, Fourier Transform Infrared Spectroscopy *(*FTIR*)* for highlighting the functional groups which are responsible for the reduction of Ag^{+} ions and capping of elemental silver, Zeta Potential for checking the stability of colloidal dispersions etc.

2.4 Egg hatch assay

Egg hatch assay (EHA) will be conducted according to the guidelines of World Association for the advancement of Veterinary Parasitology (WAAVP), (Coles *et al*., 1992). Eggs will be obtained from adult female collected from abomasum of freshly slaughtered goats at slaughter house and will be washed repeatedly with distilled water. All nanopreparations will be used as the test treatment. In addition, four controls would be prepared: untreated eggs in water will be used as a

negative control, Albendazole as a positive control, Tween 80 as the control and Tween 80 with Chitosan as the control. About 100-200 eggs in 2ml of water will be placed in the each test tube to which various concentration of test nanoformulations as well as positive and negative control would be added. The test tubes will be covered and kept at room temperature for 48 h. The number of hatched larvae (dead and alive) and unhatched eggs will be counted under microscope. The experiment will be replicated three to five times for each concentration.

2.5 *In-vitro* Assays

The adult *H. contortus* worms collected from the abomasum and placed in PBS, would be used for this assay. Ten to twenty adult actively moving worms will be placed in petri dishes containing different test concentrations and controls as with EHA. Three replicates for each treatment concentration would be employed. After 24 hours, the test solution will be washed away and the parasite will be suspended in PBS for 30 minutes for possible recovery of the parasite motility. The number of motile (alive) and immotile (dead) worms will be counted under dissecting microscope, and mortality and behaviour would be recorded for each concentration.

2.6 Cytotoxicity test (MTT assay)

Cell viability will be assessed by the (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) MTT assay (Mosmann, 1983) based on the formation of a water-insoluble formazan dye which is formed by the cleavage of the tetrazolium salt (MTT) by living (active) cells. Mammalian cell *viz.* $HepG_2$, HEK etc would be seeded in 96-well tissue culture plates using DMEM along with 10% FBS and 1% penicillin-streptomycin as a culture medium followed by an overnight incubation at 37°C temperature. The morphology of cell will be monitored with inverted microscope before exposure to nanoformulations. After 24h incubation the various test concentrations of nanoformulations will be added. Cell viability and morphology of cells will be studied after 24h, 48h and 72h of exposure. At the end of the treatment, the test concentration will be aspirated out and 10 µl tetrazolium salt solution (MTT) will be added with 90µl DMEM culture medium in each well and plate will be incubated for 4h. After 4 h of incubation at 37 °C, the supernatant from each well will be aspirated out. This procedure is used for the formation of a formazan dye by reduction of MTT through metabolically active cells. After the reduction, supernatant will be removed and 100 µl of DMSO will be added to the wells and the plates would be placed on a shaker for 20 min to dissolve formazan dye crystals. The optical density at 595 nm, for each well, will be recorded by using

microplate reader. All absorbance values would be corrected against blank wells which contained growth media alone.

2.7 Statistical analysis

All the measurements would be expressed as mean ± SD. Probit analysis will be used to determine the median (50%) lethal concentrations (LC_{50}) and 90% (LC_{90}) lethal concentrations for *in vitro* assay against adults and IC_{50} and IC_{90} for egg hatch Assay (Finney, 1971). The mortality will be corrected by Abbott's formula (Abbott, 1925) and relations between log concentration of treatment and Probit of kill will be established by probit equation. Student't' test will be used to compare mean difference between control and treated group. The data would be subjected to one way ANOVA wherever necessary.

PLAN OF WORK

Phase I

1. Synthesis of nanoformulations:
    - Preparation of biologically synthesized silver nanoparticles
    - Preparation of nanoemulsions
    - Preparation of nanoencapsulations
2. Characterization of nanoformulations through :
    - TEM, SEM, XRD, FTIR, Zeta potential analyzer etc.

Phase II

1. Evaluation of bioefficacy against *H.contortus* through :
    - Egg Hatch Assay (EHA) according to World Association for the advancement of Veterinary Parasitology (WAAVP) guidelines, (Coles *et al*., 1992).
    - *In-vitro* Adulticidal Assay

Phase III

1. Evaluation of cytotoxicity of various nanoformulations in selected mammalian cell lines through MTT assay.

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